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MASS SPECTROMETRY AND INTEGRATED VIRUS DETECTION SYSTEM CHARACTERIZATION OF MS2 BACTERIOPHAGE

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14. ABSTRACT
This study demonstrates the characterization of the sample matrix composition of the MS2 virus using ESI-MS and IVDS detection systems. The MS2 samples were grown and purified using various techniques, which showed ESI-MS samples responding differently from IVDS samples. The LC-MS of the specific biomarker of the MS2 bacteriophage from an infected *Escherichia coli* (*E. coli*) sample was characterized in the presence of *E. coli* proteins. The significant impact of the sample matrix was observed upon the identification of MS2 using a database search. *Escherichia coli* infected with MS2 showed a different score from other uninfected scores. Only purified MS2, using CsCl and analyzed by LS-MS, showed a positive match in the database search. However, the variation in the MS2 sample matrix had no effect on the characterization of MS2.

15. SUBJECT TERMS

Virus	Integrated Virus Detection System (IVDS)	Online sample processing
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EXECUTIVE SUMMARY

Electrospray ionization-mass spectrometry has been shown to be an effective tool for the characterization of viruses by analyzing their specific biomarkers. The physical properties of the viruses have been used for their characterization using an integrated virus detection system. Various experimental parameters affect the characterization of viruses by either technique in which the matrix of a virus sample is an important factor.

This approach also has the potential for several applications in basic viral research, i.e., elaborating on the effectiveness of the purification method of viral samples, and detecting genetic modification of microorganisms.

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PREFACE

The work was started in April 2005 and was completed in May 2005.

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1. INTRODUCTION

For many decades, the detection and identification of viruses have been the subjects of numerous scientific studies on viral diseases in humans, animals, and agriculture.[1-3] Rapid, accurate detection and identification of viruses are important for medical diagnoses, drug discovery, and early detection of bio-terrorism attacks on civilian installations and agricultural areas.[4-6] Several advancements in biochemical and microscopic techniques have improved the characterization of a large number of virus families.[7-12] However, these techniques have been commonly characterized as time consuming, and rely on special skills and reagents.

In proteomics, it is desirable to obtain a comprehensive mapping of the expressed proteins in an organism. Recent advances in this field have provided a promising strategy for virus characterization using protein biomarkers.[13-17] Mass spectrometry (MS) is widely used in proteomics for characterizing various microorganisms by either identifying as many protein components as possible biomarkers[15] or by identifying either one or several protein component(s) in a complex mixture.[16-17]

More MS techniques have been used to characterize bacteria than viruses.[18-21] This disparity is due to the significant difference in the number of protein biomarkers encoded in viruses compared to that of bacteria, and the thermal stability of the viral protein upon introduction into the ionization source of the MS (bacteria have a large number of proteins).[22-23]

Bacteriophage MS2 is a Leviviridae virus specific to bacteria that contain the F plasmid. The MS2 expresses four proteins:

- coat (180 copies/virion)
- lysis
- RNA-dependent polymerase
- assembly protein A (1 copy/virion) required for the maturation of the virion and pilus attachment

The large number of coat protein copies in MS2 makes it a suitable biomarker for detection and identification by mass spectrometric techniques.

Direct analysis of the intact viral capsid proteins of MS2 have been reported using ESI-time-of-flight MS.[24] Matrix Assisted Laser Desorption Ionization (MALDI) was used to analyze the viral capsid protein of MS2 and other viruses.[25] MALDI was also used to direct infusion of the MS2 bacteriophages in an *Escherichia coli* (*E. coli*) lysate into ion trap collisional

activation of intact protein ions.[26] Classical microbiological techniques, long used to characterize bacteria and viruses, are time consuming and laborious.[27] Techniques, dependent on special chemotaxonomy, were also used to characterize microorganisms by using cellular components (e.g., lipids, carbohydrates, DNA, and proteins).[28]

The demonstrated sensitivity of mass spectrometric techniques has made them increasingly attractive for accurate identification of microorganisms. A number of reports have described the use of MALDI time-of-flight (TOF) MS for bacterial and viral identification based on their DNA,[29] or proteins.[30] The ESI-MS technique has become important for analyzing viral and bacterial proteins because of its detection sensitivity and compatibility with on-line separation techniques. Viral analysis using ESI-MS has been reported with samples processed using dual microdialysis prior to either the ESI-MS analysis or direct injection of MS2 infected the *E. coli* lysate at high concentrations [1E10-1E11 plaque forming units/milliliter (pfu/mL)].

Microorganism samples often contain a wide range of impurities, cellular debris, proteinaceous components, and buffers. The ESI-MS technique has shown a limited number of buffers being used and their effect on the ionization process. Salts produce adduct that adversely affects the characterization of the desired protein biomarkers and hampers efficiency. Interference removal enhances the reproducibility of the mass spectral profile of bacterial extracts and results in a more accurate deconvolution of the mass spectra of bacterial proteins.[31,32]

Although capillary LC-ESI-MS overcomes many limitations found in MALDI analysis of microorganisms, the chromatography alone cannot eliminate all the interferences from a bacterial extract prior to ESI-MS analysis. The inability of the LC to eliminate non-proteinaceous interferences often results in ionization suppression and S/N reduction during ESI-MS analysis of bacterial proteins. Therefore, a sample pretreatment module is necessary to separate the interferential species, incompatible with LC, such as cellular debris, large particulates and nonproteinaceous components.

The Biological Sample Processing System (BSPS) is an in-house automated system designed to serve as a front end for ESI-MS interrogation of bacterial proteins.[33,34] The design of the BSPS is based on a simple, rugged, and reproducible sample-processing scheme that couples LC and ESI-MS instrumentation to provide flexibility, sensitivity, and reproducibility.

The BSPS sample pretreatment module consists of various necessary processes for an effective and reproducible bacterial analysis by ESI-MS. First, the bacterial sample is lysed to disrupt the major cell structures to release the proteins. Cell lysis is followed by the removal of cellular debris and particulates from the bacterial extract using a size exclusion filtration process. The filtrate is then subjected to sequential molecular weight filtration and extraction processes to isolate and preconcentrate bacterial proteins prior to their LC separation. The BSPS chromatographic module consists of a conventional hydrophobic capillary LC column, which is used in either the isocratic or gradient separation mode.

The physical characteristics of viruses have also been used for their characterization.[35-38] The Integrated Virus Detection System (IVDS) automates the detection and monitoring of either submicron particles or macromolecules, especially viruses and virus-like materials in many fluid types, including water and biological fluids. The detection of viruses, not limited to a particular family, genus or species, is an extremely challenging problem for current technology. The IVDS is a breakthrough in many areas. The system is simple to operate, and it performs fast, accurate, viral characterization for accurate identification and analysis.

Little attention has been drawn to the impact of impurities in viral samples, the most significant of which are the bacterial proteins in diluted viral samples. At the concentration of 1E5-1E3 cpu/mL, the number of bacterial proteins is higher than viral proteins. This difference could adversely affect virus characterization with the MS-ESI technique. Therefore, purification of viral samples from major interferences is essential for removing impurities to accurately determine the performance of subsequent detection techniques. Also, the growing concern for genetically modified microorganisms and ways to establish effective detection methods for their analyses are more reasons to pursue this study.

Various viral sample purification methods have been discussed in other studies.[39-44] This report evaluates the most conventional of the purification methods (CsCl cold fusion, ultrafiltration, and crude filtration) and explores their impact on the characterization of viruses presented at low concentrations using the LC-MS and IVDS techniques.

2. EXPERIMENTAL SETUP

2.1 Ultra Filtration of MS2.

A sample of MS2 stock solution (2 mL) was filtered with reverse osmosis water in a cross flow ultrafiltration system. This system operates by pumping the feed stream through the hollow fiber, as shown in Figure 1. A schematic of the complete flow system is shown in Figure 2. As the solution passes through the fiber, the sweeping action of the flow helps to prevent clogging. A pressure differential forces the filtrate through the fiber, while the virus feed stream is purified and concentrated. The MS2 was processed in the cross flow ultrafiltration system with a molecular weight cut-off filter (MWCO) of 100-K da. The 100-K MWCO filter allows impurities, such as growth media, miscellaneous salts, and proteins, to pass through the filter but retains the MS2. This allows the MS2 sample to be purified for further testing. After filtering with water, the MS2 solution is filtered with ~20 mL of a 20-mM ammonium acetate solution for facilitating the analysis of the test solutions in the IVDS, which requires a low concentration of low molecular weight salt solution for the electrospray injector. The MS2 was filtered until ~2.5 mL of clean stock solution remained for testing. This sample has been designated MS2-CsCl.

The remaining samples, designated MS2-ECL and MS2-ECLF, were prepared at the U.S. Army Edgewood Chemical Biological Center (ECBC) by Dr. Ilya Elashvili. These samples are discussed in detail in Sections 2.2.2 and 2.2.3.

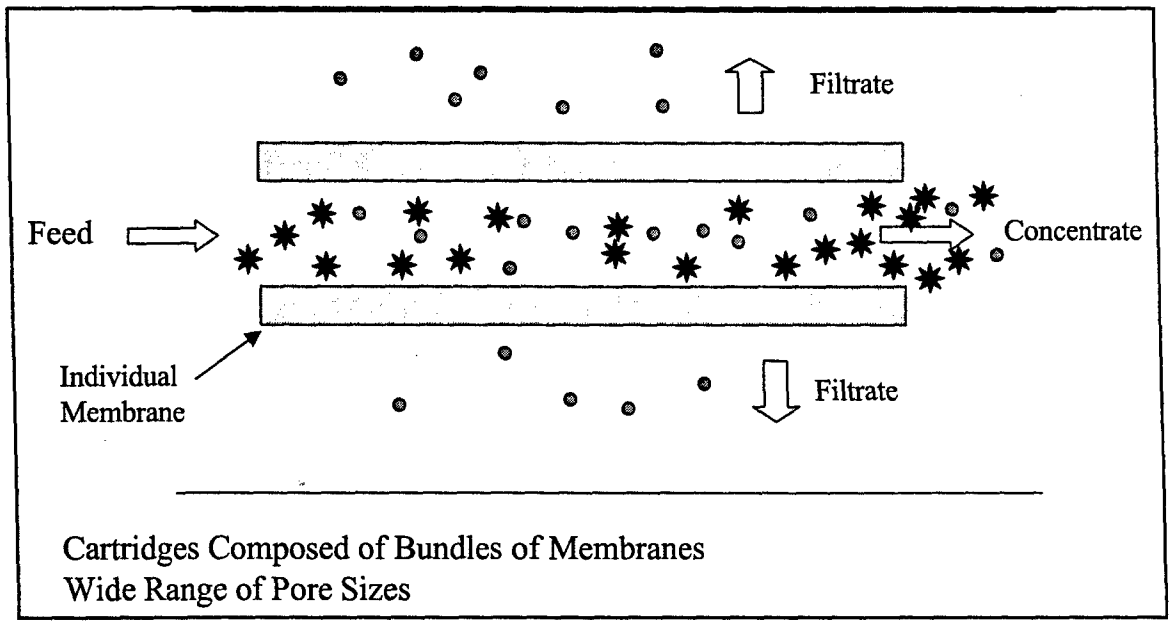

Figure 1. Individual Fiber Cross Flow Filtration

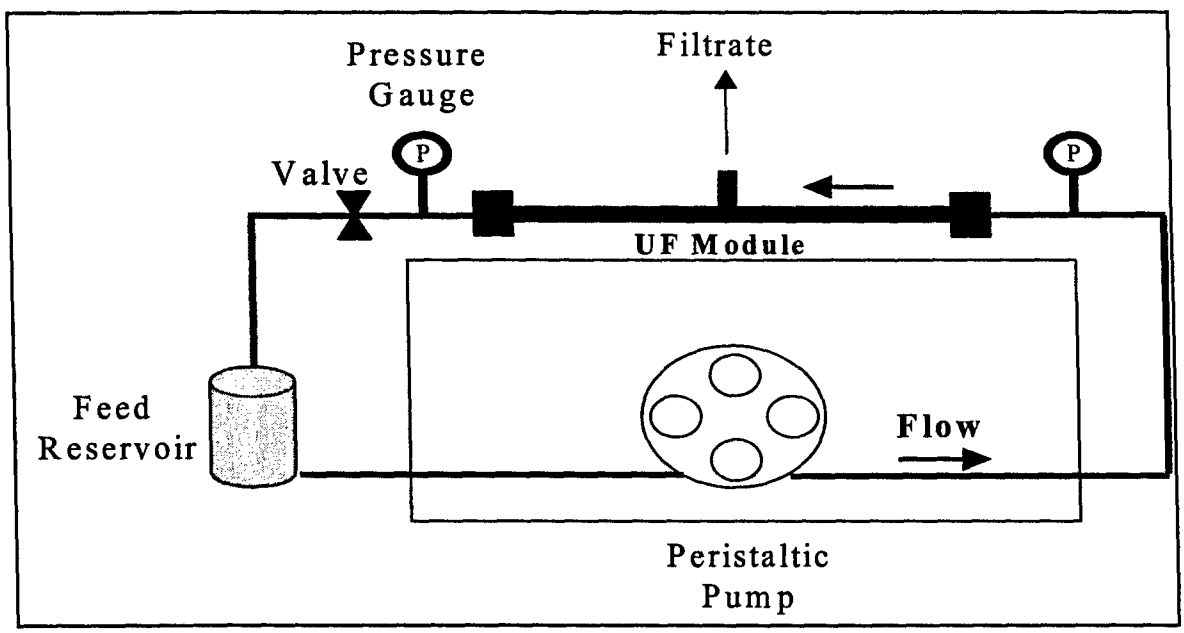

Figure 2. UF Module Using the Cross Flow Filtration Process

2.2 Experimental Procedures for MS2 Preparation.

2.2.1 Phage, Host, and Medium.

The MS2 (ATCC 15597-B1) bacteriophage and its *E. coli* (ATCC 15597) host strain were obtained from the American Type Culture Collection (Manassas, VA). *E. coli* strain 15597 was grown on #271 broth (Difco, USA), which according to Davis and Sinsheimer[45] contained (per liter):

- 10 g of tryptone
- 8 g of NaCl
- 1 g of yeast extract
- 0.1% of glucose
- 2 mM of $CaCl_2$
- 10 μg/mL of thiamine hydrochloride

2.2.2 Preparation of MS2 Bacteriophage.

The protocol for the MS2 phage preparation was modified from Strauss and Sinsheimer.[46] To limit the amount of uninfected dead bacteria, the procedure was conducted in two stages. The starter, fresh phage lysate was prepared by inoculating 2 mL of #271 broth with a single colony of an overnight culture of *E. coli* ATCC 15597 strain and then incubating the mixture at 37 °C in an incubator-shaker operating at 200 rpm. After 90 min, the mixture was added to 100 mL of prewarmed #271 broth in a 250-mL Erlenmeyer flask and incubated at 37 °C at 200 rpm. When the growth reached A_{600} of 0.15, MS2 bacteriophage was added from the frozen stock at a multiplicity of infection ca. 3, and the incubation was continued. After lysis occurred (4 hr), the lysate was chilled at 4 °C and centrifuged at 2,800 rpm (ca. 1500 x g) for 10 min at 4 °C. The resulting supernatant was filtered through a sterile 0.2 μm filtration unit. The filtrate was collected into a sterile bottle as the starter MS2 phage lysate and was kept at 4 °C until used for the next stage of large-scale MS2 phage preparation.

For the large-scale growth of MS2 phage, 2 L of #271 MS broth was inoculated with a 40-mL overnight culture of *E. coli* ATCC 15597 strain in a 6-L Erlenmeyer flask and incubated at 37 °C in an incubator-shaker operating at 200 rpm. When the growth reached A_{600} of 0.17, 2 mL of starter MS2 phage lysate was added, and incubation continued until lysis occurred (<2 hr). The lysate was chilled at 4 °C and centrifuged at 2,800 rpm (ca. 1500 x g) for 10 min at 4 °C. The resulting supernatant was labeled "MS2-ECL."

Portion (ca. 220 mL) of MS2-ECL was filtered through a 0.2-μm filter, and the resulting filtrate was labeled "MS2-ECLF."

2.2.3 Ultra Filtration of MS2 Bacteriophage.

Purified MS2 phage was isolated by using cesium chloride equilibrium gradient, which was modified from the protocol described by Sambrook and Russell.[47] The cesium chloride was dissolved in TSM medium, before being subjected to ultra-centrifugation at 23 °C. Following centrifugation, the samples were transferred to Slide-a Lyzer (Pierce, Rockford, IL) and dialyzed in 500-mL TSM for 24 hr with 2 buffer changes.

Samples MS2-ECL and MS2-ECLF were prefiltered through a 0.2-μm filter to remove large impurities (e.g., cell fragments and debris). The samples were then filtered with reverse osmosis water in a cross flow ultrafiltration system, using the 100-K MWCO filter described in Section 2.1.

2.3 IVDS Description.

The MS2-CsCl, MS2-ECL, and MS2-ECLF samples were analyzed using the IVDS. The detection stage of the IVDS consists of an electrospray unit to inject samples into the detector, a Differential Mobility Analyzer (DMA), and a Condensate Particle Counter (CPC).

The electrospray unit subjects a conductive liquid to a strong electric field. The field produces a cone that emits a fine jet, which breaks up into small droplets and forms a fine plume. To eliminate the possibility of the breakdown (corona discharge) of the air in the plume, caused by a high electric field, the spray tip is surrounded by a CO_2 flow, which prevents corona discharge.

The DMA separates particles by their electrical mobility in the air. The sample stream flows through a gap between a rod and a cylinder with an electrical potential between the two. Particle mobility, which is related to size and charge, either passes particles through the DMA or impinges on the walls. With singly charged particles, which are generated by the electrospray, the mobility becomes a direct measure of the particle size.

In the CPC, the sample particles flow in tandem with a saturated working butanol fluid. The nanosized particles initiate the butanol condensation, and the stream is cooled. A standard optical counter is used to count the butanol-condensed particles, and the supplied software is used to display the results.

A complete description of the IVDS system, including the detector, can be found ERDEC-TR-453.[38]

2.4 SDS/Polyacrylamide Gel Electrophoresis.

All MS2 samples were subjected to SDS/polyacrylamide gel electrophoresis as a final visual check of purity, which was performed according to the method described by Laemmli et al.[48] Commercially available precast 18% SDS polyacrylamide gels (Tris-Glycine gels) for the Novex gel apparatus system were purchased from Invitrogen (Carlsbad, CA) and used according to manufacturer's instructions. The Tris-Glycine SDS-PAGE running buffer and

sample buffers were either purchased from Invitrogen (Carlsbad, CA) or made according to manufacturer's instructions. Samples were diluted by 50% (v/v) in 2X Tris-Glycine sample buffer, incubated at 85 °C for 2 min, and then directly loaded on the gels. Electrophoresis was carried out for 2-3 hr at 30-40 mA/gel. The gels were stained in Brilliant Blue R solution (Sigma, St. Louis, MO) according to the manufacturer's instructions and destained in a 30% methanol:10% acetic acid:60% (v/v) water solution for 8 hr.[49]

2.5 BSPS Parameters.

Figure 3 provides a general overview of the BSPS modules and analysis procedures applied to biological samples. The sonication method is integrated into the BSPS. Twenty microliters of a lysed bacterial sample was subjected to a Microcon©-3 Kda size exclusion membrane from Millipore (MA, USA). The Microcon© has a MWCO of 3 Kda. After size exclusion filtration, the bacterial extracts were transferred to a hydrophobic C18 or C8 protein micro-trap cartridge from Michrom (CA, USA). Bacterial proteins from the liquid phase were captured onto a small band of solid phase in the trap to provide preconcentration and purification of the bacterial proteins. The bacterial proteins were eluted in a 20-50-μL sample volume from the protein micro-trap cartridge into the LC module using a 6-valve LC port transfer system. The reverse phase (RP) LC columns were either hydrophobic silica based C18 or C8 and were purchased from Michrom (CA, USA) and Phenomenex (CA, USA). The RPLC system used either a gradient or an isocratic mode of separation. The aqueous phase was 95/5/0.1-1% H_2O/ACN/AA, FA, or TFA, and the organic phase was 90/10/0.1-1% ACN/H_2O/ AA, FA, or TFA.

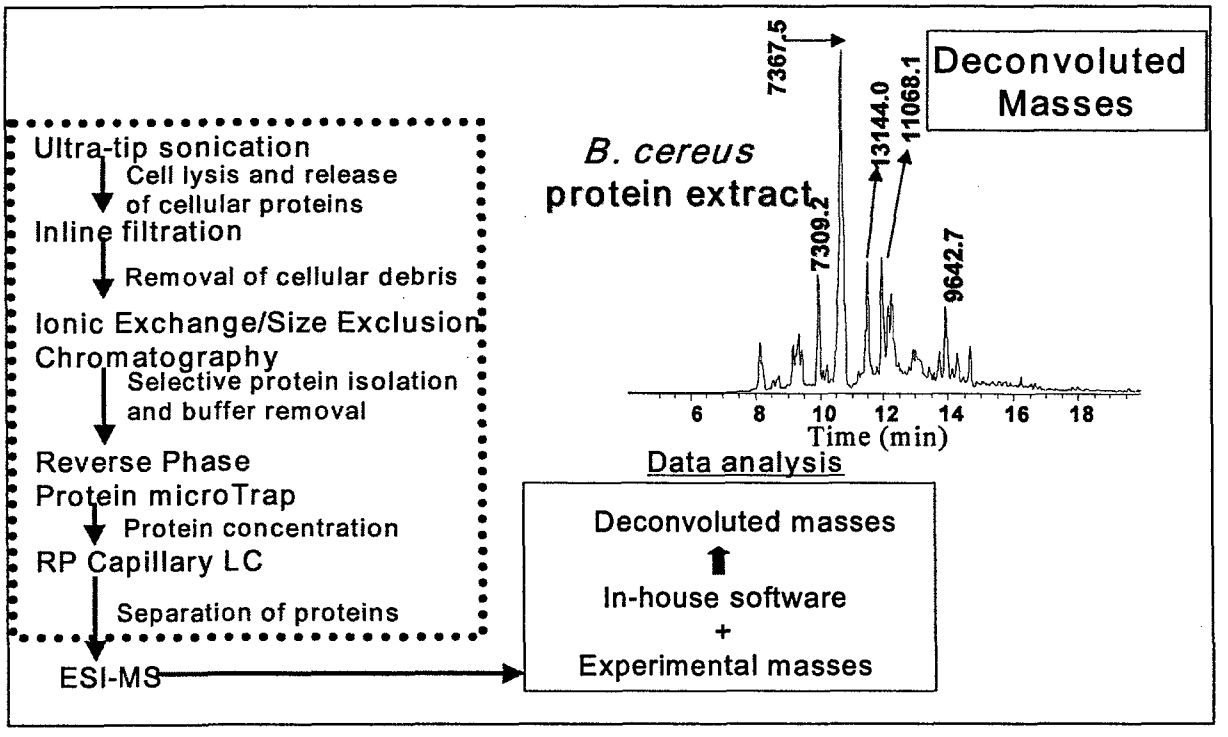

Figure 3. Overview of BSPS Modules and Analysis Procedures

2.6 ESI-MS Parameters.

An ion trap mass spectrometer (LCQ-Deca, Thermo Finnigan-USA) equipped with an ESI ion source was used. The mass spectrometer was operated under the control of the X-Caliber program with a manual deconvolution algorithm. Spectra were collected in the positive ion mode. Three microscans were used with a maximum ion injection time of 200 ms. The ESI spray voltage was maintained at 4 kV. The capillary voltage was maintained at 23 V, and the temperature of the ion transport tube was 190 °C. The mass spectrometer was calibrated to achieve a ±2-da resolution using a mixture of cytochrome c, BSA, insulin, and myoglobin proteins.

2.7 Automated Deconvolution Algorithm Analysis.

The automated in-house deconvolution algorithm was developed to provide a filtered mass list instead of a conventional peak-at-every-mass output for the BSPS-MS analysis of bacterial extracts. Briefly, the in-house software deconvolutes the bacterial protein masses through analysis of a raw mass spectral file. Mass range, isotope peak width, S/N threshold, and a maximum number of returned peaks user input parameters were selected prior to the start of the deconvolution process. The software identified LC peaks and deconvoluted their corresponding average mass spectra to generate a list of masses. The total deconvolution process takes 10 min for a 60-min BSPS-ESI-MS analysis. The deconvolution process is interfaced with relational database management software to update the in-house database with experimental bacterial protein masses.

3. RESULTS AND DISCUSSION

3.1 IVDS Results.

The IVDS detector analyzes the solution containing the MS2 bacteriophage and displays the results in a scan showing the numerical MS2 counts versus the particle size in nanometer. Figure 4 is the scan of the sample, MS2-CsCl. Figure 5 is the scan of MS2-ECLF, and Figure 6 is the scan of MS2-ECL. All output graphs are an average of three scans each.

3.2 MS Results.

The ESI-MS analysis is ideal for determining the effectiveness of the sample purification approach. The purification of viral samples from bacterial lysate is important for the direct analysis of a virus by ESI-MS. The presence of bacterial lysate in a viral sample while a virus protein marker is analyzed at a low concentration range (1E5-1E3 pfu/mL) could hinder virus characterization using ESI-MS. Bacterial genome, for instance *E. coli*, is expressed around 4000 bacterial proteins with a few thousand to hundreds of thousands da molecular weight range. Thus, sensitivity considerations for the analysis of a virus by ESI-MS dictate that viral proteins be present in relatively higher concentrations and a low molecular weight range (5-50 Kda). Using BSPS interfaced with the ESI-MS provided the removal of much of the possible interference prior to the ESI-MS analysis from low molecular weight proteinaceous components in the cell lysate to the buffer and salts in the purified viral solution.

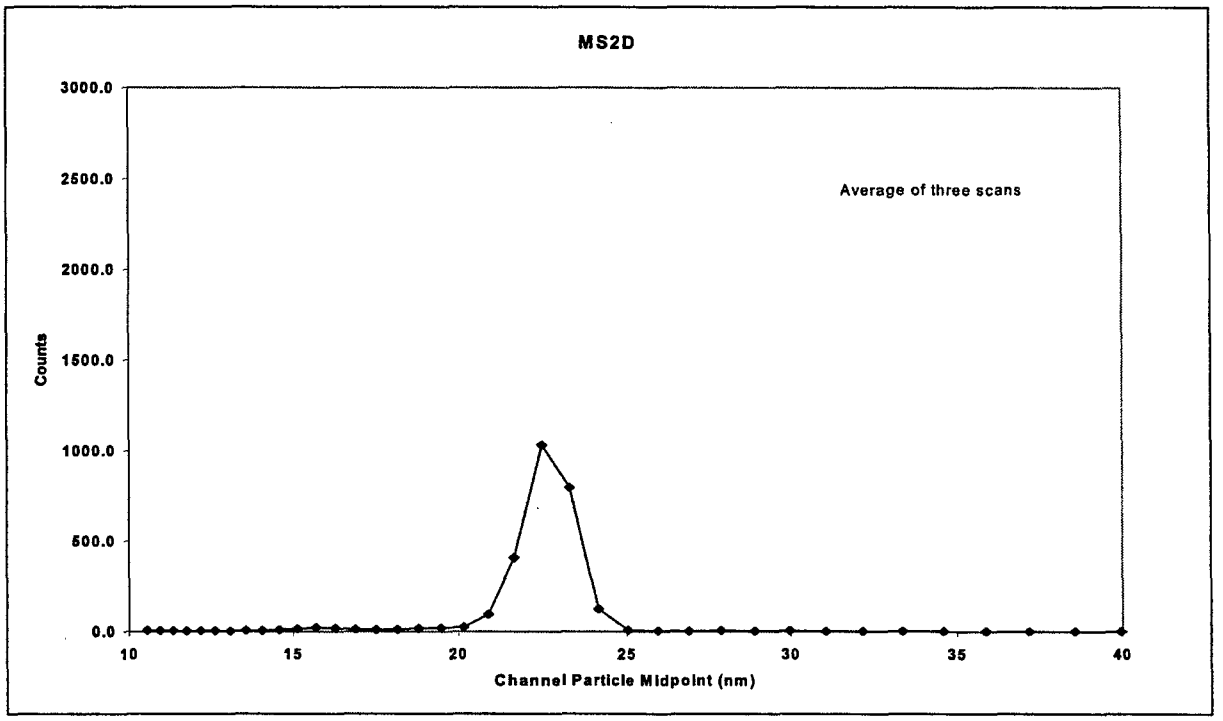

Figure 4. IVDS Scan of MS2-CsCl

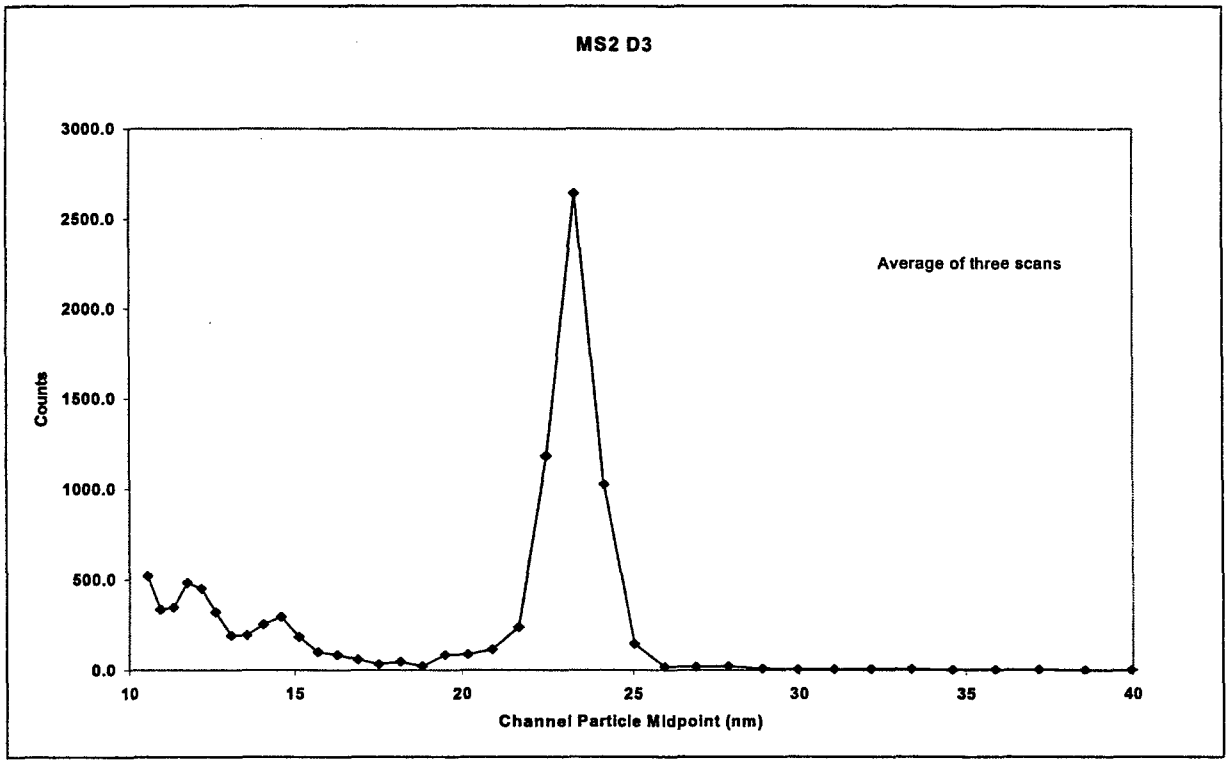

Figure 5. IVDS Scan of MS2-ECLF

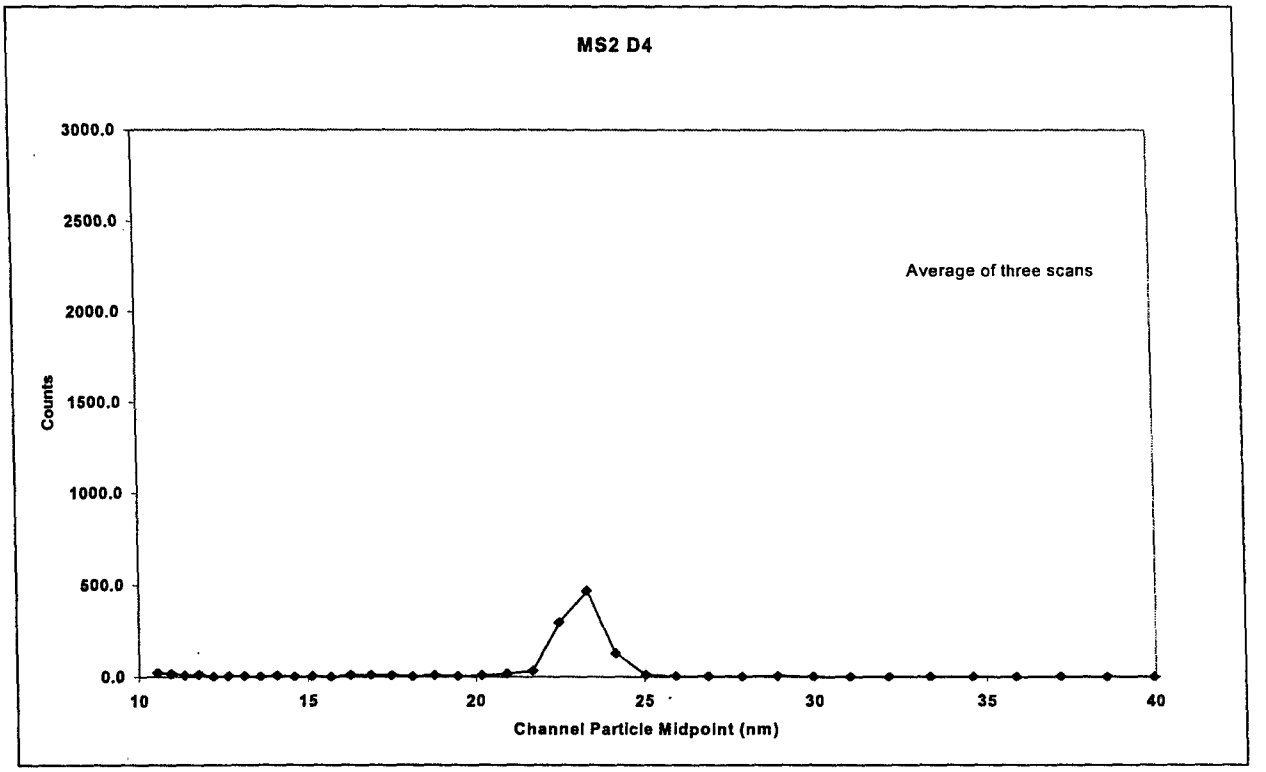

Figure 6. IVDS Scan of MS2-ECL

Three MS2 samples, collected from different purification protocols, were used to demonstrate the virus characterization capabilities of the BSPS-ESI-MS and IVDS. The MS2 samples were obtained from different purification approaches. The first MS2 sample, MS2-CsCl, was purified using the CsCl purification approach. The second MS2 sample, MS2-ECL, was collected from crude *E. coli* lysate. The third MS2 sample, MS2-ECLF, was collected from crude *E. coli* lysate and then purified using a 0.2-μm polyvinylidene fluoride (PVDF) filtration membrane to remove cellular debris. All of the MS2 samples were then diluted from 1×10^{10} pfu/mL to 1×10^{4} pfu/mL and analyzed using the BSPS-ESI-MS and IVDS.

The total ion chromatogram (TIO) plot of the MS2-CsCl sample analyzed using BSPS-ESI-MS is shown in Figure 7a. The graph shows two distinct peaks with S/N > 5 and retention times of 30.04 and 32.06 min. The mass spectrum of the relatively major peak at 33.30 min is shown in Figure 7b, where the characteristic m/z ratios of the MS2 markers are observed upon mass spectrum deconvolution. The resulting molar mass of this MS2 protein was calculated as 13,728 ± 1.2 da (Figure 7c), which corresponds to the reported molecular weight of the MS2 coat protein (Swissprot reference). The deconvolution of the minor peak observed at 31.05 min was 44,248 ± 3.4 da, which also agreed with the accepted molecular weight for the MS2 A protein.

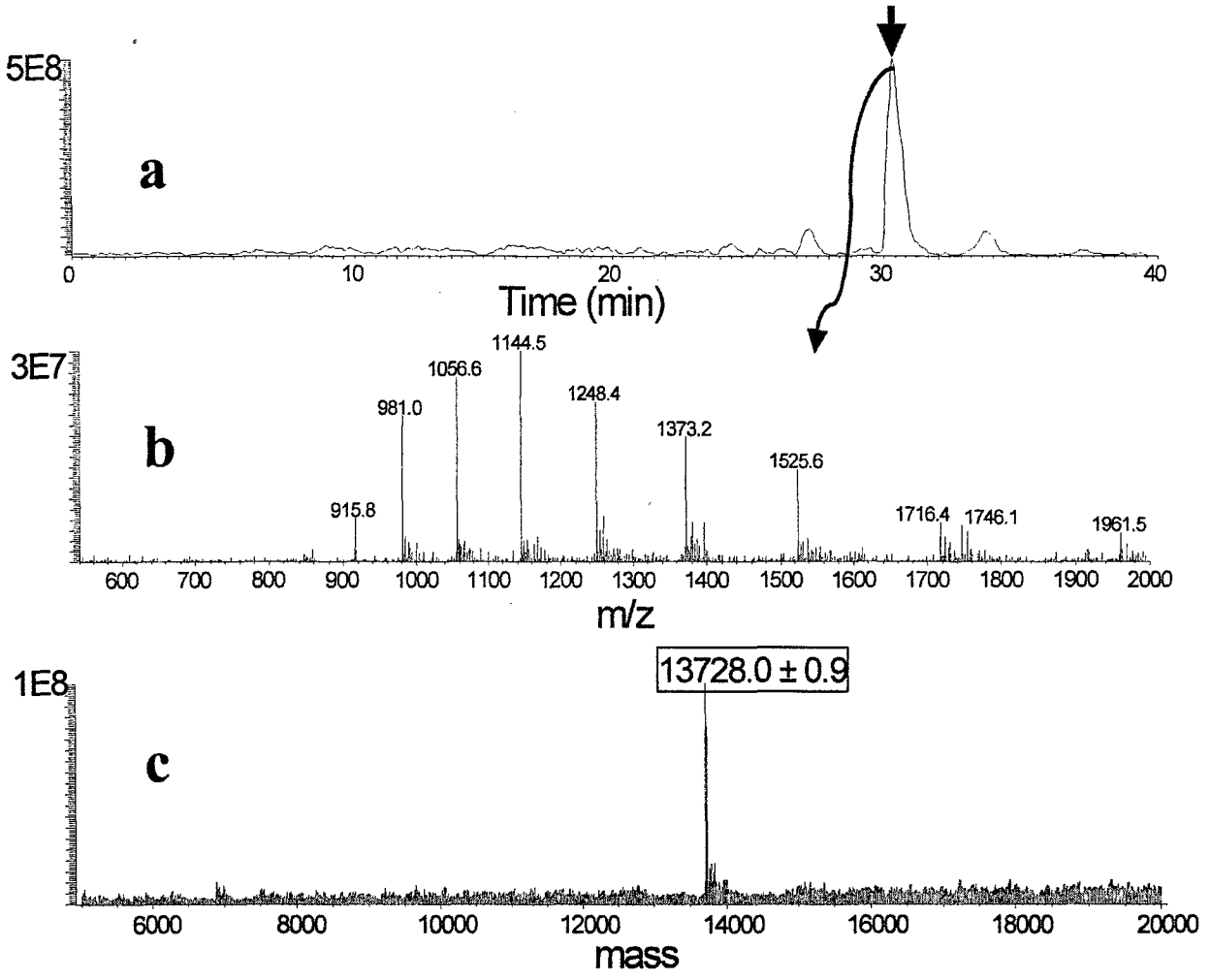

Figure 7. MS2 Sample Purification Using CsCl Purification Approach

Experimental Conditions: Luna C8 column, 300 A, 50 x 0.3 mm; flow rate was 10 mL/min, mobile phases: 98/2/0.05-0.1 H_2O/ACN/acid additive for A, 95/5/0.05-0.1 ACN/H_2O/acid additive for B; 2-98% B in 30 min; concentration of protein injected was 0.1 pmole/mL; injection volume was 10 mL.

The observed masses were supported by the analysis of the MS2 sample using 1-D-gel electrophoresis as shown in Figure 8. In the figure, the most intense protein bands are observed in the molecular range of 14,000 da with low intensity bands in the 44,000-da range. Figure 8 also shows a comparison between the 1-D-gel analyses of two MS2 samples from different purification methods. The MS2 with *E. coli* lysate clearly shows that the extra bacterial protein bands were not observed with the MS2-CsCl sample. This indicates that ultrafiltration of the MS2 samples using a 100-K da filter membrane did not remove the bacterial proteins of *E. coli*, which could be due to its large size (> 100-K da) and the number of bacterial proteins.

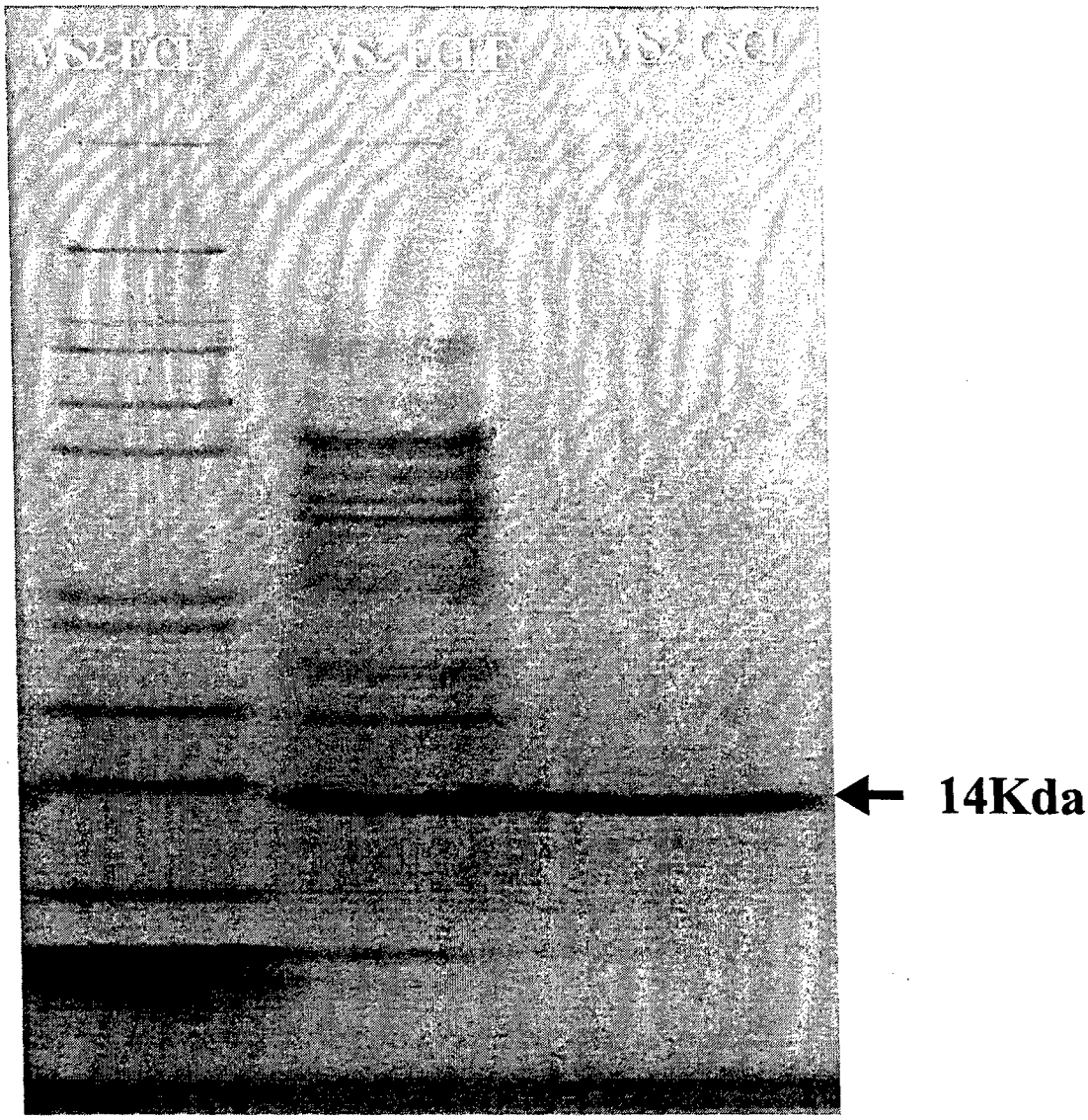

Figure 8. 1-D-Gel Analysis of the Three MS2 Samples

The two MS2 samples obtained from the *E. coli* lysates (MS2-ECL and MS2-ECLF) were analyzed using the BSPS-ESI-MS and IVDS techniques. Figures 9 and 10 show the TICs obtained from the replicate BSPS-ESI-MS analyses of the MS2-ECL and MS2-ECLF, respectively. The retention times of the MS2 coat protein in MS2-ECL and MS2-ECLF samples were observed around 26.30 and 25.60 min, respectively.

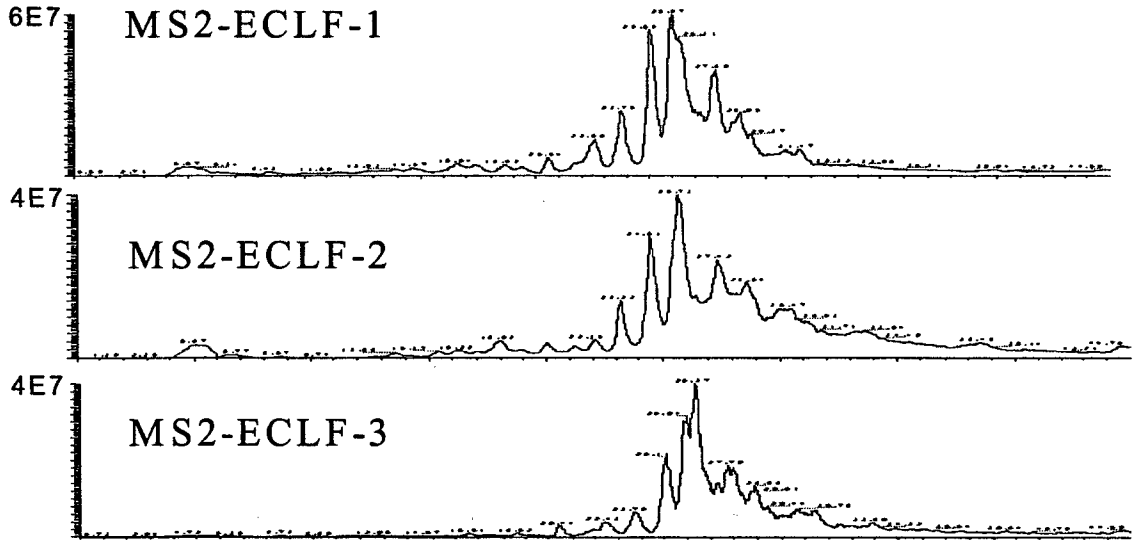

Figure 9. Replicate TIC Plots of Three Separate MS2-ECLF Samples
All three samples were collected from the same growth harvest and subjected to the same experimental conditions.

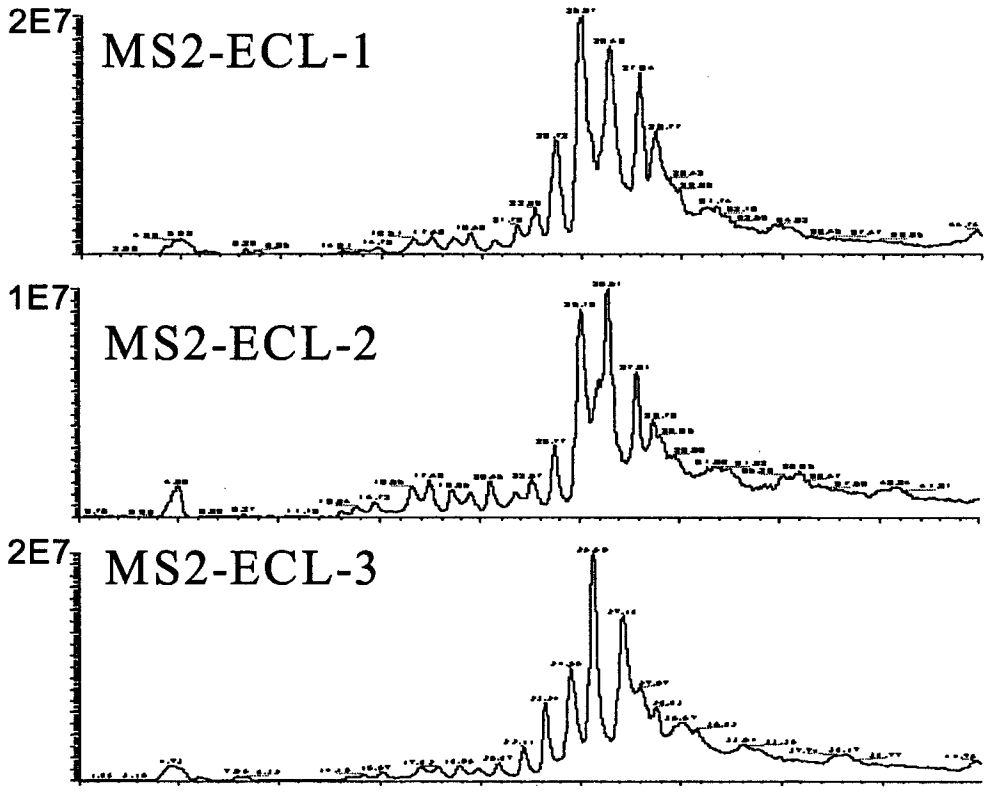

Figure 10. Replicate TIC Plots of Three Separate MS2-ECL Samples
All three samples were collected from the same growth harvest under the same experimental conditions.

Figures 9 and 10 show that the TICs of these MS2 samples are characterized by the presence of a larger number of peaks than those observed with the MS2-CsCl samples in Figure 7, which indicates the presence of bacterial proteins in the MS2 sample. Moreover, the relative signal intensity of the MS2 coat proteins decreased as the number of peaks increased in the TIC plots of bacteriophage MS2 lysates as seen in Figures 9 and 10. The decrease in signal intensity is an indication of the effect of the presence of variable amounts of bacterial proteins on the ionization efficiency of low concentrations of the MS2 virus (1E5-1E3 pfu/mL) in samples MS2-ECL and MS2-ECLF. This assumption is supported by the fact that a satisfactory signal intensity of the MS2 coat protein was present at roughly the same concentration level, with no evidence of bacterial protein (Figure 7a-c) for the MS2 purified by the CsCl method.

A comparison of the retention time of the MS2 coat protein obtained for the BSPS-ESI-MS and the MS2-CsCl analyses is shown in Figure 11. This figure presents the extracted ion chromatogram for the MS2 coat protein from the three different MS2 samples. The retention time for the MS2 coat protein from the analysis of the MS2-CsCl, MS2-ECL, and MS2-ECLF samples was observed at 31.00, 25.30, and 25.10 min, respectively. This figure shows that the protein biomarker obtained from the BSPS-ESI-MS analysis of the MS2-CsCl sample, which relatively contains the least concentration of impurities than that of MS2-ECL and MS2-ECLF, had the longest retention time than the biomarkers of the other two MS2 samples. Figure 11a-c shows how the purification of the MS2 samples not only affected the mass spectrometric characteristics but also the chromatographic behavior of the MS2 coat protein. Since the mode of separation was based on the reverse phase, the presence of *E. coli* bacterial proteins induced less available retention sites for the MS2 coat protein. Thus, as bacterial protein concentrations increased, the retention time of the MS2 coat protein decreased.

Sample purification also affected virus identification through use of protein content as a biomarker. To examine this observation further, the deconvolution of the mass spectra, observed for the three different MS2 samples, was generated using an in-house automated deconvolution algorithm. Figure 12 shows a histogram of the common masses of the bacteriophage MS2 lysate generated from the deconvolution of the mass spectra from Figures 7, 9, and 10. Visual comparison of the obtained molecular mass pattern for the MS2 showed obvious differences between MS2-CsCl, MS2-ECL, and MS2-ECLF. The number of the dominant masses observed with the MS2-CsCl sample were characterized by having the MS2 coat protein as the dominant mass and the MS2 protein A (molecular mass: 42,242 ± 1.8 da) as the lesser mass. However, in the deconvolution of the TIC plots for the MS2-ECL and MS2-ECLF samples, the signal intensity of the MS2 coat protein was overshadowed by the presence of other bacterial proteins, with equal or larger signal intensity, and by the presence of a larger number of bacterial protein masses than the MS2-CsCl sample. Although, the molecular weight of the MS2 coat protein was accurately determined, the observed mass accuracy alone cannot be used for an unambiguous virus identification. A combination of protein mass deconvolution with statistical database correlation and peptide mass fingerprinting could provide the necessary tools for unequivocal virus identification. Since the objective of this study is to determine the effect of sample purification on the characterization of the MS2 virus by BSPS-ESI-MS and IVDS, more attention will be devoted to conventional database searches using protein masses and statistical correlation between the number of masses in the experimental database versus number of matches in the public and in-house experimental databases.

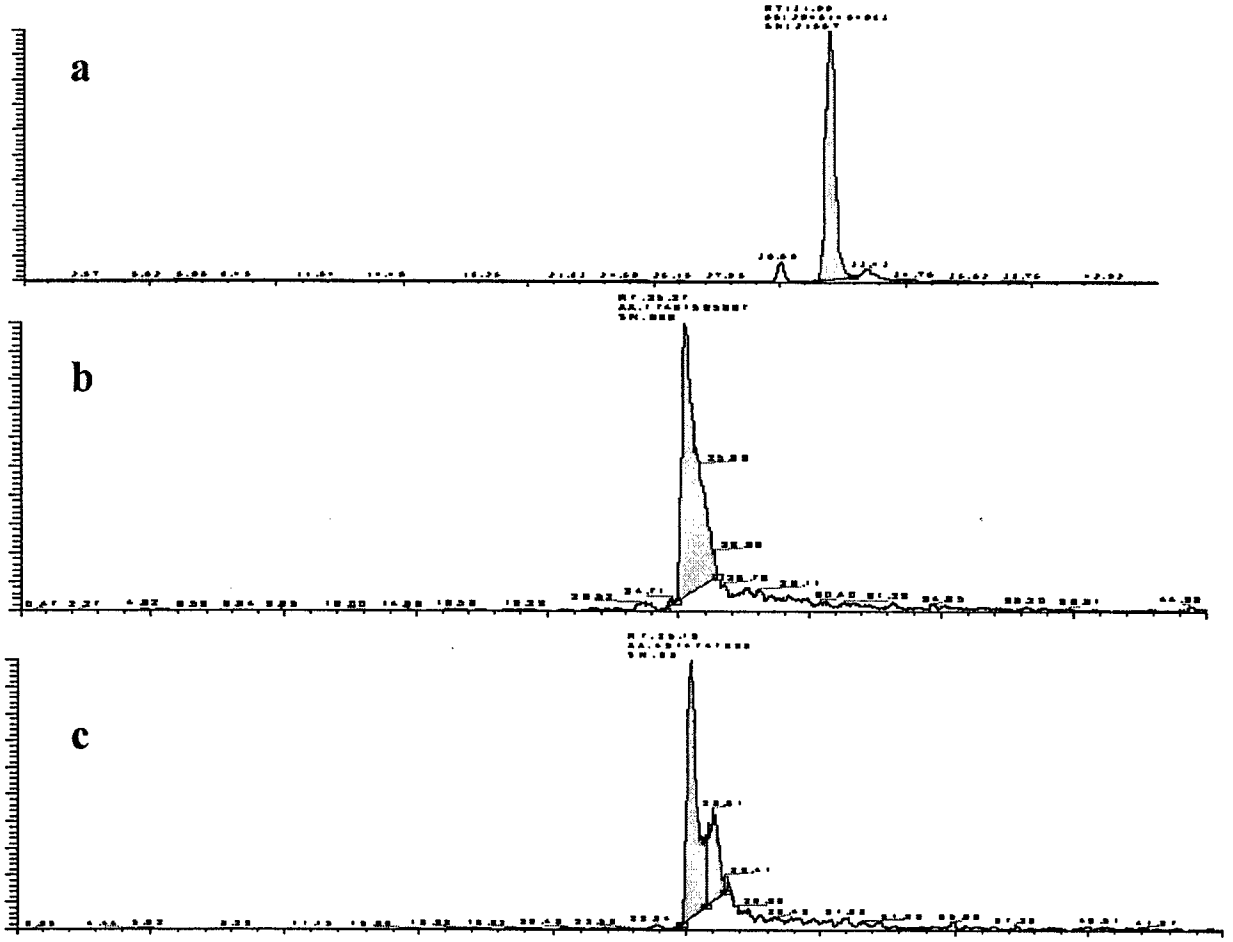

Figure 11. Comparison of Ion Extracted Chromatogram of MS2 Coat Protein from MS2 Samples

Figure 11a presents the ion extracted chromatogram of MS2 biomarkers for the MS2-CsCl. In Figure 11b, the ion extracted chromatogram of MS2 biomarkers for the MS2-ECLF are shown, and Figure 11c presents the ion extracted chromatogram of MS2 biomarkers for the MS2-ECL.

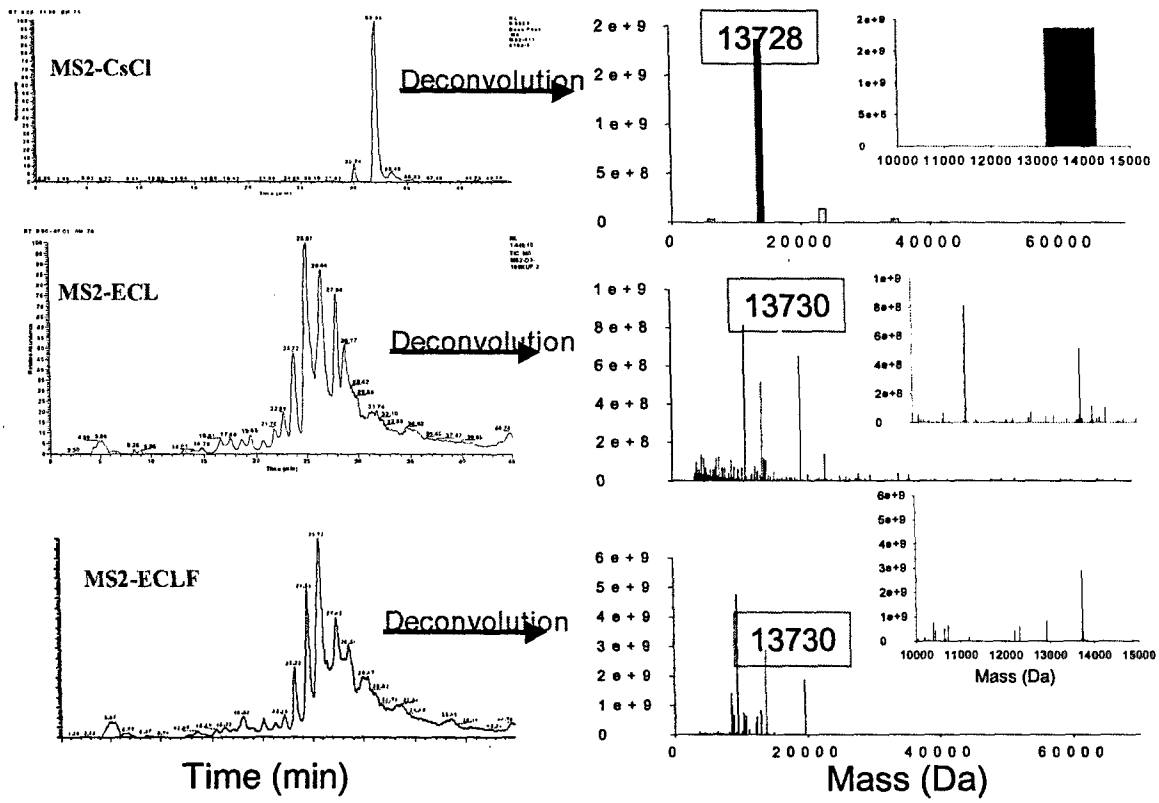

Figure 12. Deconvolution of the TIC Plots for MS2 Samples

The inset graph represents the deconvoluted masses between 10 Kda - 15 Kda.

Comparing only the molecular weight of the MS2 coat protein with the proteome masses in the public database resulted in variable identification of the MS2 virus. Using only the calculated molecular weight of the MS2 coat protein, correct MS2 identification was achieved for the MS2-CsCl sample with a calculated molecular weight of 13728 da. However, correct MS2 identification could not be achieved for the MS2-ECLF and MS2-ECL samples with calculated molecular masses of 13729 and 13730 da, respectively. Instead, these samples showed the highest match with F2 and R17 viruses, which was expected given the fact that the public database contains a large collection of proteome databases for various types of microorganisms.

The bacterial genome for several bacteria expressed a large number of proteins with a wide range of molecular weights falling within the same range as the MS2 coat protein, leading to multiple matches with several microorganisms. The Table shows the results of a database search for MS2 virus identification for scoring matches in the calculated molecular masses listings of MS2 samples. Modification of the search criteria was implemented to enhance the distinct identification of the MS2 virus, where a scoring coefficient representing the number of experimental mass entries verses that of the number of matches in the proteome database is used in the final scoring match. A reference sample of *E. coli* 0157 was analyzed and used as reference to verify the validity of the approach. Accordingly, the mass list from the purified

MS2 sample showed the primary match with MS2 (92%) followed by *E. coli* (15%), while the mass lists from the other two MS2 samples showed the highest matching score correlation range was with the *E. coli* proteome (80-90%), while their correlation with the MS2 proteome was ranked fourth with a range of 30-35% scoring match.

Table. Identification of MS2 Results from the Scoring Match of the Deconvoluted Mass Lists of the MS2 Samples with the SWISS-PROT/TrEMBL Proteome Database

Sample ID	Search Qualifier	No. of Deconvoluted Masses	Database: SWISS-PROT/TrEMBL Scoring Match @ Mass Tolerance (±2 Da)	
			MS2 (%)	*E. coli* 0157 (%)
MS2-CsCl	MW	6	92	15
MS2-ECLF	MW	52	35	82
MS2-ECL	MW	95	32	90
E. coli O57	MW	412	46	95

On the other hand, the MS2 infected *E. coli* lysates have a larger number of deconvoluted protein masses matching the *E. coli* proteome database than those of the MS2-CsCl sample. This high correlation, 90-95% matching score, serves as an independent reference to the origin of these deconvoluted masses. Accordingly, most of the protein masses in these samples resulted from the *E. coli* lysis.

4. CONCLUSIONS

Bacterial proteins, present in diluted viral samples, adversely affected the identification of MS2 in a protein masses database search. The purification of viral samples using CsCl cold fusion was determined to be the most suitable purification method to characterize MS2 using the mass spectrometry-electrospray ionization (MS-ESI) technique. The sensitivity of MS-ESI determined the appropriate method for viral sample purification. Direct MS-ESI analysis of genetically modified bacterial samples using this approach did not show a significant discrimination with nonmodified bacteria. The Integrated Virus Detection System (IVDS) showed MS2 peaks without the interference of the *Escherichia coli* protein masses. The IVDS did not show any other viral materials in the scanned ROI. A 10-to 100-nm scan was displayed from 10 to 40 nm for peak clarity. This system could be used as a preliminary technique to scan for viruses present in a given bacterial sample because it has less affinity to the impurities present in viral samples.

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APPENDIX

MS2 STRUCTURAL INFORMATION

Virus	Type	Proteins	Size	Comments
Leviviridae	MS2 bacteriophage	Two structural virion proteins found. Protein size 35000-44000 da. Capsid contains one copy of A protein, which is required for maturation of the virion and the pilus attachment. Protein size of 2nd largest 14000 da. Coat protein; capsid contains 180 copies of the coat protein, arranged in 60 identical triangular units.	Virions not enveloped. Nucleocapsids isometric; 24-26 nm in diameter. Symmetry icosahedral. 32 capsomers per nucleocapsid.	Molecular mass (Mr) of virion $3.6\text{-}4.2 \times 10^6$ (depending on the genus). Buoyant density 1.46 g cm^{-3} in CsCl. Sedimentation coefficient 80-84 S. Virions sensitive to detergents. Virions not sensitive by di-ethyl ether and chloroform. Infectivity reduced after exposure to irradiation.

PDB-ID : 2MS2
Resolution : 2.8 Å
A.A.Seq.Acc.# : P03612
Family : Leviviridae
T Number : 3
# of Subunits : 180
Diameter: Ave: 268Å; Max:288Å

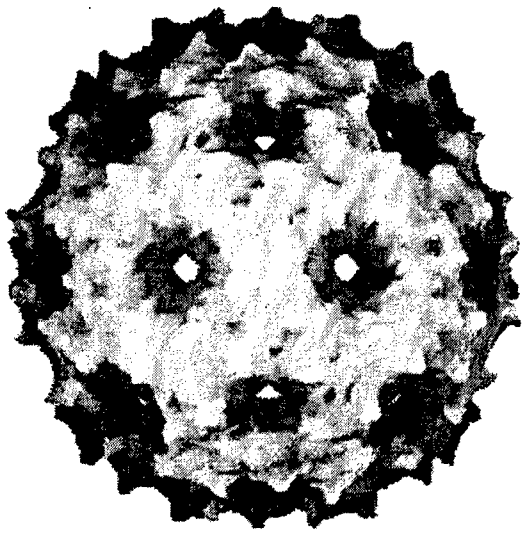

Figure A-1. Rendered Surface

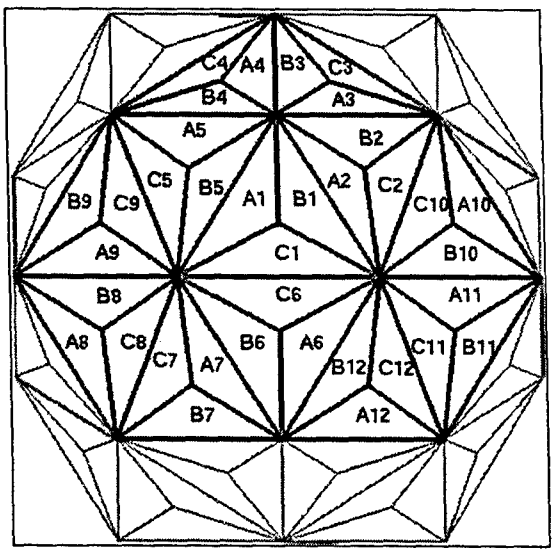

Figure A-2. Diagram of the Tertiary Folding Constituent Subunits in MS2

T= 3 Lattice

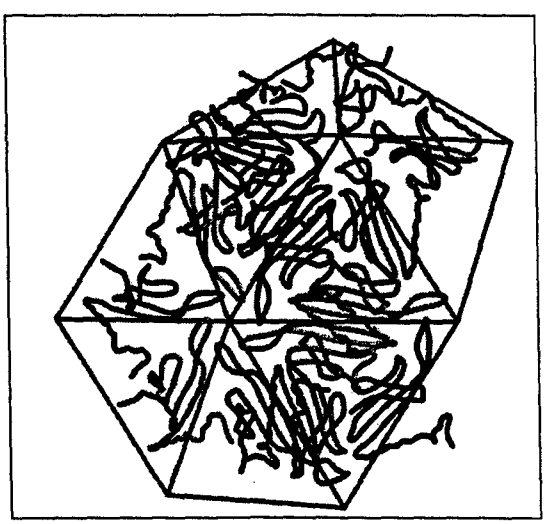

Figure A-3. MS2 Capsomeres

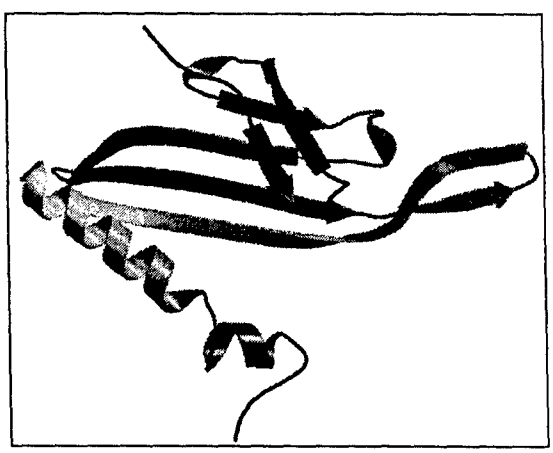

Figure A-4. N-Terminus (Blue) to C-Terminus (Red) MS2 Protein Subunits

Reddy et al., (2001). Virus Particle Explorer (VIPER), a Website for Virus Capsid Structures and their Computational Analysis. J. Virol. 75:11943-11947.)